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Saturation mutagenesis reveals the importance of residues αR145 and αF146 of penicillin acylase in the synthesis of β-lactam antibiotics

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Abstract

Penicillin acylase (PA) from *Escherichia coli* can catalyze the coupling of an acyl group to penicillin- and cephalosporin-derived β-lactam nuclei, a conversion that can be used for the industrial synthesis of β-lactam antibiotics. The modest synthetic properties of the wild-type enzyme make it desirable to engineer improved mutants. Analysis of the crystal structure of PA has shown that residues αR145 and αF146 undergo extensive repositioning upon binding of large ligands to the active site, suggesting that these residues may be good targets for mutagenesis aimed at improving the catalytic performance of PA. Therefore, site-saturation mutagenesis was performed on both positions and a complete set of all 38 variants was subjected to rapid HPLC screening for improved ampicillin synthesis. Not less than 33 mutants showed improved synthesis, indicating the importance of the mutated residues in PA-catalyzed acyl transfer kinetics. In several mutants at low substrate concentrations, the maximum level of ampicillin production was increased up to 1.5-fold, and the ratio of the synthetic rate over the hydrolytic rate was increased 5–15-fold. Moreover, due to increased tendency of the acyl–enzyme intermediate to react with β-lactam nucleophile instead of water, mutants αR145G, αR145S and αR145L demonstrated an enhanced synthetic yield over wild-type PA at high substrate concentrations. This was accompanied by an increased conversion of 6-APA to ampicillin as well as a decreased undesirable hydrolysis of the acyl donor. Therefore, these mutants are interesting candidates for the enzymatic production of semi-synthetic β-lactam antibiotics.

Keywords: Penicillin acylase; Antibiotic synthesis; Ampicillin; Directed evolution; Protein engineering; Mutagenesis

1. Introduction

The β-lactam family of antibiotics, including penicillins, is the most important class of antibacterial compounds in clinical application. The narrow bactericidal spectrum of naturally occurring penicillin G, its low acid stability, and emerging resistance problems have triggered the development of semi-synthetic penicillins since the late 1940s, leading to the introduction of ampicillin in 1961 (Bergan, 1984; Levy, 1998; Nayler, 1991). Nowadays, ampicillin is one of the most widely used semi-synthetic β-lactam antibiotics with an estimated market of 20,000 tonnes year^{-1} (Bruggink, 2001). Its chemical synthesis is done under harsh conditions using reactive intermediates and organic solvents at a low temperature, causing high downstream processing costs and processes that are environmentally undesirable (Bruggink et al., 1998). Therefore, biocatalytic production processes for semi-synthetic antibiotics are highly desirable. The first step of such a process involves the PA-catalyzed cleavage of penicillin G into phenylacetic acid (PAA) and the penicillin nucleus 6-aminopenicillanic acid (6-APA). In the second step, 6-APA is coupled to a phenylglycine side group, yielding ampicillin. This second step can also be catalyzed by PA if an ester or amide of phenylglycine is used (Bruggink et al., 1998; Kasche, 1986; Alkema et al., 2002a; Youshko et al., 2000). However, the synthetic capacities of known PAs are only modest and need to be improved for economically

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competitive usage in large-scale production of penicillins and cephalosporins.

The enzyme-catalyzed synthesis of β-lactam antibiotics can be carried out in either an equilibrium-controlled or a kinetically controlled conversion (Svedas et al., 1980a,b; Kasche, 1986). In an equilibrium-controlled conversion the product concentration cannot be influenced by the properties of the enzyme as the enzyme only affects the rate at which conversion occurs. The level of product accumulation that can be reached is governed by the thermodynamic equilibrium, which is unfavorable in case of ampicillin synthesis (Svedas et al., 1980a; Schroen et al., 1999). In a kinetically controlled conversion, however, the enzyme catalyzes the transfer of the acyl group from the activated acyl donor to a nucleophilic acceptor (6-APA or a cephalosporin-derived nucleus). For the preparation of semi-synthetic penicillins, the acyl donor is usually the amide or methyl ester of an aromatic carboxylic acid. In this case, the level of product accumulation is governed by the catalytic properties of the enzyme and high non-equilibrium concentrations of the acyl-transfer product can transiently be obtained (Svedas et al., 1980b; Youshko et al., 2002a,b). The ability of PA to catalyze effective acyl transfer to β-lactam antibiotic nuclei is very much dependent on the reaction conditions (Ferreira et al., 2004; Ospina et al., 1996; Park et al., 2000), and the type of PA and therefore can be influenced by mutating the enzyme's active site (Alkema et al., 2002b; Gabor and Janssen, 2004).

Both structural and kinetic data have shown that PA catalyzes the conversion of amides and esters via an acyl–enzyme intermediate, in which residue Ser1 of the β-subunit is esterified to the acyl group (Duggleby et al., 1995; Konecny et al., 1983). Either the amino group of an added external nucleophile (6-APA) or water can attack the acyl enzyme, yielding the desired acyl-transfer product (antibiotic) or the hydrolyzed acyl donor, respectively. The ratio between the rate of synthesis, v_{Ps} and rate of hydrolysis, v_{Ph}, is an important parameter for evaluating the synthetic performance of PA. Since the initial value of this ratio, $(v_{Ps}/v_{Ph})_{ini}$, or the so-called synthesis/hydrolysis ratio (Youshko et al., 2001, 2002a), is dependent both on the kinetic properties of the enzyme and the concentration of the nucleophilic acceptor (i.e. 6-APA), it can be used to compare different enzyme variants. The maximum level of product accumulation that is transiently achieved is a second important parameter that is used for comparison of PA variants.

Youshko et al. (2002a) have shown that the course of acyl transfer is predicted by three enzyme-dependent parameters, α, β_0 and γ, as well as initial concentrations of acyl donor and nucleophile (β-lactam nucleus). The $(v_{Ps}/v_{Ph})_{ini}$ is hyperbolically dependent on the nucleophile concentration of the nucleophile (nucleus) according to Eq. (1):

$$\left(\frac{v_{Ps}}{v_{Ph}}\right)_{ini} = \frac{\beta_0[Nu]}{1+\beta_0\gamma[Nu]} \quad (1)$$

The hyperbolic dependence reflects a situation in which the acyl–enzyme complex that is formed during PA-catalyzed acyl transfer can still be hydrolyzed by water even if nucleophile is bound to it, e.g. at saturating nucleophile concentrations. Under these saturating conditions, the $(v_{Ps}/v_{Ph})_{ini}$ reaches a maximum value $(1/\gamma)$. Thus, γ should be low for good synthesis. The parameter β_0, which represents the preference of the acyl enzyme to react with nucleophile instead of water, should be high. The relative preference of the free enzyme for synthetic product versus the acyl donor is expressed with the specificity parameter α (Eq. (2)), which describes competition between two substrates (acylated β-lactam antibiotic and acyl donor) for the enzyme. It should obviously be low.

$$\alpha = \frac{(k_{cat}/K_m)_{Ps}}{(k_{cat}/K_m)_{AD}} \quad (2)$$

These three parameters describe the concentration of the β-lactam antibiotic during the course of the conversion according to Eq. (3).

$$\frac{d[Ps]}{d[Ph]} = \frac{\beta_0[Nu][AD] - \alpha[Ps](1+\beta_0\gamma[Nu])}{(1+\beta_0\gamma[Nu])([AD]+\alpha[Ps])}, \quad (3)$$

with $[AD]_0 = [AD] + [Ps] + [Ph]$ and $[Nu]_0 = [Nu] + [Ps]$, where [AD], [Nu], [Ps] and [Ph] are, respectively, the concentrations of acyl donor, nucleophile, product of synthesis (antibiotic) and product of hydrolysis. $[AD]_0$ and $[Nu]_0$ are the initial concentrations of acyl donor and nucleophile.

The selectivity of the enzyme for the nucleophile is governed by the active-site geometry. Done et al. (1998) reported that the crystal structure of PA can adopt two distinct and energetically favored conformations, the open and closed form. A 16 amino acid long α-helix becomes interrupted between residues αM142 and αA143, allowing a movement of the last part of the helix upon substrate binding. The closed conformation (helical state) is adopted in the ligand-free enzyme or if a small ligand occupies the substrate-binding site, whereas after binding of larger ligands, such as 3,4-dihydrophenylacetic acid and *m*-nitrophenylacetic acid, the enzyme is in the open conformation (coil form). In the latter conformation, residues αM142–αF146 are repositioned towards the solvent and the α-helix is partially unfolded. Upon binding of penicillin G to the inactive mutant βN241A, in which the oxyanion hole is corrupted because it is partly formed by the side chain oxygen of N241, the enzyme also adopts the open conformation (Alkema et al., 2000; McVey et al., 2001). In the helical form, αR145:NH_2 is hydrogen bonded to the main chain carbonyl oxygen of residue βF24. Upon binding of penicillin G, this hydrogen bond is replaced by one between an oxygen atom of the carboxylate group of the ligand and αR145:NH_2 via two bridging water molecules (Alkema et al., 2000), as residue αR145 orients itself into the solvent and residue αF146 moves 3.5 Å towards the solvent (Fig. 1). Such intricate changes may influence the pK_a values of groups close to the active site, which is important for catalysis (Morillas et al., 1999).

The extensive repositioning of αR145 and αF146 upon substrate binding as well as the interactions between these residues and the bound β-lactam ring prompted us to investigate the effect of mutations at these positions on the synthetic properties of penicillin acylase. For example, the intricate structural changes could well influence nucleophile binding and reactivity as well as sensitivity of the acyl–enzyme intermediate towards hydroly-

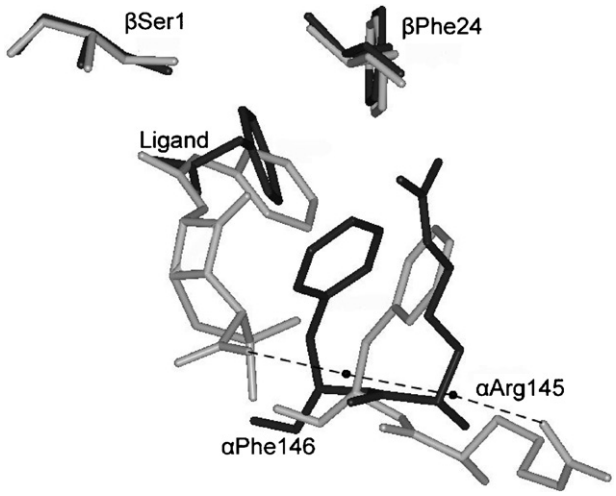

Fig. 1. The position of residues αR145 and αF146 in the substrate-binding site of penicillin acylase. The structure of the inactive mutant βN241A (light grey), containing penicillin G in the active site, is superimposed on the wild-type PA structure, shown in black. Residues βS1 and βF24 have not shifted, while residues αR145 and αF146 are repositioned upon binding of penicillin G and the hydrogen bond between residue αR145 and βF24 is replaced by a water-bridged hydrogen bond between residue αR145 and the substrate.

sis. To probe possible effects on the catalytic performance of PA, we have constructed a set of all possible single mutants on positions αR145 and αF146. For the characterization of the mutants, a rapid screening method was used, employing an automated 96-well pipetting station that was connected to a HPLC. Of the 38 mutants, 33 displayed enhanced synthetic performance in synthesis of ampicillin, of which the most promising ones were mutants αR145G, αR145S and αR145L. The properties of these mutants are presented.

2. Materials and methods

2.1. Strains and plasmids

The gene encoding PA of *Escherichia coli* used by us holds two mutations, causing a mutation in the spacer region and a βV148L substitution in the mature protein as compared to Swiss-Prot entry P06875 derived from *E. coli* ATCC 11105.

2.2. Mutagenesis

The desired mutations were introduced using the Quickchange site-directed mutagenesis kit of Stratagene (La Jolla, US) in a microtiter plate format. The 27 nucleotide primers contained the codon that was to be mutagenised and 12 nucleotides on either side of this codon. The forward primer used to introduce the mutation on position α146 was 5′-ATG.GCA.AAC.CGC.xxx.TCT.GAT.AGC.ACT-3′. The xxx represents the codon that was used to introduce the specific mutation. The reverse primer for this position was the 27-mer 5′-AGT. GCT.ATC.AGA.yyy. GCG.GTT.TGC.CAT-3′, in which codon yyy is the reverse complement of codon xxx. For position α145 the 27-mer 5′-ACC.ATG.GCA.AAC.xxx.TTC.TCT. GAT.AGC -3′ was used. The xxx stands for the codon that was used to introduce the specific mutation on position αR145. As reverse primer the 27-mer 5′-GCT.ATC.AGA.GAA.yyy.GTT.TGC.CAT.GGT-3′ was used. The following codons were used: A, gcc; C, tgc; D, gac; E, gag; F, ttc; G, ggc; H, cac; I, att; K, aag; L, ctc; M, atg; N, aac; P, ccc; Q, cag; R, cgc; S, tcc; T, acc; V, gtc; W, tgg; Y, tac.

DNA amplification was done using 0.5 μl *Pfu* polymerase (Stratagene) in a volume of 25 μl. The reactions were performed with a thermocycler (Hybaid Ltd., Ashford, UK) employing the following program: 0.5 min 94 °C, 18 cycles of 0.5 min at 94 °C, 1 min at 55 °C and 14 min at 72 °C, followed by 5 min at 72 °C. After amplification the reaction mixture was incubated with 10 units DpnI at 37 °C for 2 h. Competent *E. coli* cells (50 μl) were transformed with the ligation mixture (5 μl) in 96-well microtiter plates (MTPs) and the plates were incubated on ice for 30 min. A heat shock was given by incubating the MTPs for 2 min at 42 °C, after which the plate was incubated at 0 °C for another 2 min. Then 250 μl LB medium was added to each well and the plate was incubated in a shaker at 37 °C for 1 h. Subsequently, 150 μl cells were plated on LB plates containing 68 mg l^{-1} chloramphenicol. For each mutant, four transformants were transferred to MTPs containing 250 μl of LB medium with 68 mg l^{-1} chloramphenicol. These plates were incubated overnight at 37 °C under shaking conditions at 200 rpm and stored at −80 °C after addition of glycerol to a final concentration of 10%.

2.3. Screening for improved mutants

Synthetic activities of mutants stored in MTPs were determined by transferring 10 μl cell suspension to a second MTP, containing 240 μl LB medium supplemented with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 68 mg l^{-1} chloramphenicol. The cells were allowed to grow for 36 h at 16 °C and 200 rpm, after which the cells were centrifuged at 2500 rpm in a MSE Mistral 2000 MTP centrifuge. The cells were resuspended in 200 μl reaction mixture containing 15 mM D-phenylglycine amide (PGA) and 10 mM 6-APA in 50 mM phosphate buffer, pH 7.0. All MTP liquid handling was done using a Plato3001 automated pipetting station (Rosys AG, Switzerland) that was equipped with an automatic HPLC sampler. Samples were taken at various times and analysed by HPLC, using an Alltima C18 rocket column in connection with a Jasco PU-1586 pump and a Jasco UV-1586 detector set at 214 nm. All compounds were eluted isocratically using an aqueous solution containing 680 mg l^{-1} SDS and 30% acetonitrile in 5.0 mM phosphate, pH 3.0, which quenches the reaction. Concentrations of phenylglycine (PG) and ampicillin were determined and the ratio of the ampicillin concentration over the PG concentration ($[P_s]/[P_h]$-ratio) was calculated and compared to the value of the wild-type enzyme.

Periplasmic extracts were prepared as follows. The cells were harvested by centrifugation at 6000 rpm for 10 min, resuspended in 0.5 volume of ice-cold osmotic shock buffer A (20% sucrose, 100 mM Tris–HCl, pH 8.0, 10 mM EDTA), and centrifuged at 3600 × *g* for 10 min. The pellet was resuspended in 0.02 vol-

Table 1
Properties of the mutants and wild-type enzyme as determined by rapid screening[a]

Mutant	αF146			αR145		
	Time[b] (h)	$[P_s]/[P_h]_{obs}$	[Ampicillin] (percentage of wild type)	Time (h)	$[P_s]/[P_h]_{obs}$	[Ampicillin] (percentage of wild type)
WT	1.5	0.3	100	1.5	0.3	100
G	24	1.2	61	1.5	1.7	256
A	7.5	2.50	99	1.5	1.6	154
V	24	0.8	153	1.5	1.6	127
L	24	3.7	169	1.5	0.9	237
I	7.5	4.8	135	1.5	1.1	120
M	24	1.3	85	1.5	1.5	129
C	7.5	3.7	241	1.5	1.7	169
F	–	–	–	4	3.8	131
W	24	<0.05	10	4	5.3	103
Y	1.5	<0.05	0	4	1.9	37
T	24	1.3	376	4	1.5	127
S	24	1.5	238	1.5	1.8	192
N	24	1.2	151	1.5	1.0	173
Q	24	1.7	114	1.5	0.7	158
D	24	<0.05	0	4	0.9	15
E	24	0.1	13	4	0.4	6
K	48	1.4	120	1.5	2.6	145
R	24	<0.05	3	–	–	–
H	1.5	1.0	174	4	2.3	78
P	48	2.7	112	1.5	0.6	137

[a] Reaction conditions: 15 mM PGA and 10 mM 6-APA in 50 phosphate buffer, pH 7.0.
[b] Time indicates the sampling time at which the highest level of ampicillin was observed.

ume of ice-cold osmotic shock buffer B (1 mM EDTA) and centrifuged at 3600 × *g* for 10 min. Potassium phosphate buffer (1 M, pH 7.0) was added to the resulting periplasmic extract to a final concentration of 50 mM. The enzyme concentrations of the periplasmic extracts and pure enzymes of the αR145 mutants and mutant αF146H were determined using phenylmethylsulfonylfluoride (PMSF) titration (Svedas et al., 1977; Alkema et al., 1999). The conversion experiments were carried out at 30 °C in 50 mM potassium phosphate buffer, pH 7.0. The reaction was started after addition of the substrates 6-APA and D-PGA to a final concentration of 25 and 15 mM, respectively. Samples were taken and analysed by isocratic HPLC, using a Chrompack C18 column in connection with a Jasco PU-980 pump and a Jasco UV-1575 detector set at 214 nm.

The kinetic parameters β_0 and γ were determined by performing several synthesis experiments, using 50 nM enzyme solution, 15 mM PGA and 6-APA in the range of 1.25–35 mM in 50 mM phosphate buffer, pH 7.0. The kinetic parameter α was obtained by determining the steady state kinetic parameters k_{cat} and K_m for PGA and ampicillin hydrolysis using HPLC. The data were fitted using the program SigmaPlot.

2.4. Chemicals

PMSF was from Serva (Heidelberg, Germany). Both 6-APA and D-phenylglycine amide were a gift from DSM-Gist (Delft, The Netherlands) and ampicillin was obtained from Sigma.

3. Results

3.1. Synthetic performance of αF146 penicillin acylase mutants

Penicillin acylase of *E. coli* undergoes a conformational change of residues αM142–αF146 upon binding of larger ligands to the active site of PA (Done et al., 1998; McVey et al., 2001; Alkema et al., 2000, 2002b). This process may influence the β-lactam binding site, making residues αR145 and αF146 reasonable candidates for mutagenesis aimed at improving the synthetic properties of this key enzyme. Consequently, we have constructed all 38 single mutants by site-directed mutagenesis.

Cells of the αF146 mutants and the wild-type enzyme were grown in a microtiter plate, after which the mutants were tested for their synthetic performance using a rapid screening method. In this method, ampicillin is synthesized from 15 mM PGA as the acyl donor and 10 mM 6-APA as the acyl acceptor, and at four times samples of the incubation mixtures were analyzed for product formation by HPLC. Formation of ampicillin and PG was determined and the $[P_s]/[P_h]$ was calculated. The resulting values were compared to the highest level of *E. coli* wild-type PA. Most mutants showed improved synthetic properties but at the same time had a lower synthetic (and hydrolytic) activity than the wild-type enzyme, causing the highest ampicillin concentration to be reached later. The ratio between the concentrations of ampicillin and phenylglycine that was found when the ampicillin level reached its highest value was

Table 2
Kinetic constants for ampicillin synthesis of the αF146 mutant PAs obtained with periplasmic extracts

Mutant	$[P_s]_{max}$[a] (mM)	$(v_{Ps}/v_{Ph})_{ini}$
E. coli wild type	2.2	1.4
αF146G	>2.5[b]	2.5
αF146V	2.9	>2.2
αF146L	2.9	4.5
αF146I	3.2	6.5
αF146C	2.8	2.2
αF146H	2.8	>4.7
αF146P	>1.7[b]	5.1
αF146Q	3.3	3.5

[a] Reaction conditions: 15 mM PGA and 25 mM 6-APA in 50 mM phosphate buffer, pH 7.0.
[b] The maximum product concentration was not reached within 2.5 h.

called $[P_s]/[P_h]_{obs}$ and it appeared to be significantly altered in the mutants as compared to wild-type PA (Table 1). Thus, the residue at position α146 greatly influences the deacylation selectivity. Thirteen mutants showed an elevated $[P_s]/[P_h]_{obs}$ value, of which 11 mutants produced more ampicillin than the wild-type enzyme. Especially high values were seen for mutants αF146S and αF146T. Also a hydrophobic residue at this position had a positive effect on synthesis. A negative charge on position α146 caused a strong decrease of the synthetic performance, whereas the effect of a substitution by a positively charged residue varied, since mutant αF146R showed a decreased $[P_s]/[P_h]_{obs}$, while that of mutant αF146K appeared to be increased. Unfortunately, this was always accompanied by a strong reduction of the synthetic activity as compared to the wild-type enzyme, implying that very long reaction times were needed for a high level of product accumulation. The mutants required 7.5–24 or even 48 h to obtain a level of ampicillin production that the wild-type enzyme already reached in 1.5 h. Only mutant αF146H retained the synthetic activity of the wild-type enzyme. The hydrolytic activity also appeared to be very low for the α146 mutants, except for mutant αF146Y, which showed an increase in the hydrolytic activity, which is in agreement with previously reported results (Alkema et al., 2000).

Table 3
Kinetic constants for ampicillin synthesis of the αR145 mutants, obtained with periplasmic extracts[a]

Mutant	$[P_s]_{max}$ (mM)	$(v_{Ps}/v_{Ph})_{ini}$	Synthetic activity (percentage of wild type)
E. coli wild type	2.2	1.4	100
αR145A	2.4	2.1	88
αR145Q	2.7	1.4	71
αR145P	3.0	3.4	61
αR145L	2.9	5.2	44
αR145T	3.2	4.8	36
αR145V	2.9	3.5	33
αR145N	2.9	3.0	30
αR145G	3.3	5.7	29
αR145Y	3.1	3.7	26
αR145S	3.5	4.6	21
αR145C	3.4	5.0	20
αR145M	3.4	3.7	18
αR145I	2.9	2.4	16
αR145K	3.1	4.8	11
αR145F	3.4	6.5	9
αR145W	3.5	14.3	6
αR145H	3.1	3.6	5

[a] Reaction conditions: 15 mM PGA and 25 mM 6-APA in 50 mM of phosphate buffer, pH 7.0.

3.2. Synthetic performance of the αR145 mutants

Mutants were also constructed at position αR145. This residue moves out during binding of a ligand with large substituents on the aromatic group of a substrate that binds in the acyl binding pocket (McVey et al., 2001). Remarkably, it was found that for most of the αR145 mutants the cultivated cells contained a higher amount of active enzyme than the cultures expressing the wild-type enzyme. This could indicate that the maturation of the mutants, a process that is believed to be autocatalytic (Hewitt et al., 2000), is facilitated in the α145 mutants due to the altered active site, an observation that was not found for the mutants on position αF146. All mutants at position αR145 showed a more effective synthesis (higher $[P_s]/[P_h]_{obs}$) than wild-type PA (Table 1), and the reduction of synthetic activity that was observed for the αF146 mutants was not seen for the αR145 mutants. Moreover, mutants αR145G, αR145L, αR145S

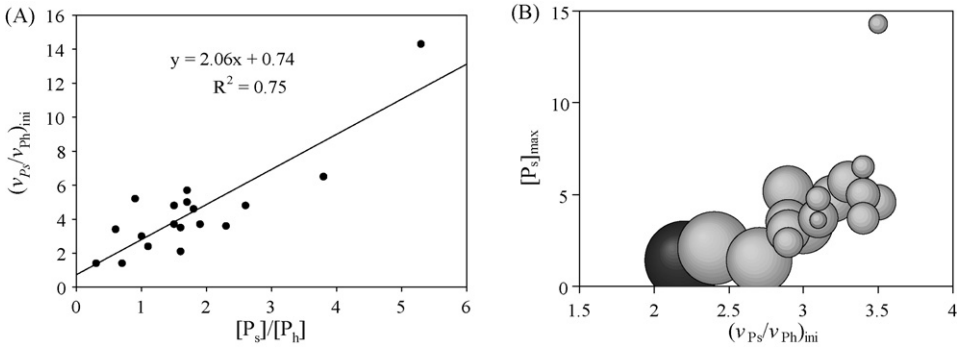

Fig. 2. (A) Correlation between the $[P_s]/[P_h]$-ratio, determined in the rapid screening method and the $(v_{Ps}/v_{Ph})_{ini}$ in the second screening. (B) Correlation between the rate of synthesis (v_{PS}), represented by the size of the spheres, the maximum level of product accumulation and the initial synthesis hydrolysis ratio. The values of the wild-type enzyme are shown in dark grey.

Table 4
Kinetic constants for ampicillin synthesis of the αR145 mutants obtained with purified enzyme

Enzyme	$[P_s]_{max}$[a] (mM)	$(v_{Ps}/v_{Ph})_{ini}$[a]	Synthetic activity[a] (percentage of wild type)	α[b]	β_0[b] (M^{-1})	γ[b]
WT	2.2	1.4	100	7.7	0.08	0.14
α145G	3.6	7.2	28	29	0.42	0.04
α145S	3.3	6.2	16	14	0.35	0.05
α145L	2.8	4.8	42	15	0.28	0.06

[a] Reaction conditions: 15 mM PGA and 25 mM 6-APA in 50 mM phosphate buffer, pH 7.0.
[b] Reaction conditions: 15 mM PGA and 1.25–35 mM 6-APA in 50 mM phosphate buffer, pH 7.0.

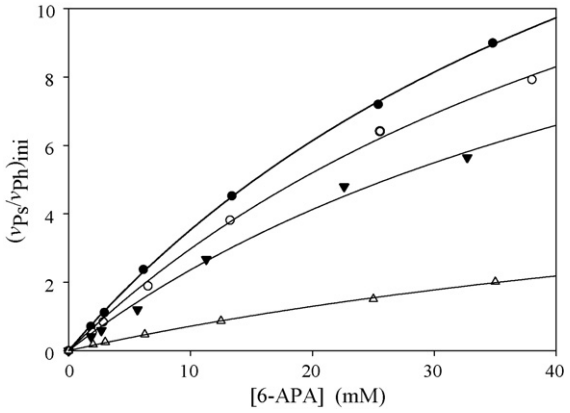

Fig. 3. Nucleophile reactivity of 6-APA in the synthesis of ampicillin by wild-type PA and the three selected α145 mutants. The PGA concentration was 15 mM. *E. coli* PA (Δ), mutant αR145L (▼), mutant αR145S (◯) and mutant αR145G (●). The lines were fitted using Eq. (1).

and αR145C produced ampicillin to much higher levels than wild-type PA. Only when Arg145 was replaced by a negatively charged amino acid, hardly any ampicillin was produced. Further analysis was done using periplasmic enzyme extracts, using only mutants with improved synthetic performance and reasonable rate.

3.3. Initial rates and correlation with product accumulation

Based on the observed improved values for $[P_s]/[P_h]_{obs}$, the most promising mutants were selected for a more detailed characterization using incubations with periplasmic extracts to determine the $(v_{Ps}/v_{Ph})_{ini}$ and $[P_s]_{max}$ values (Tables 2 and 3). The 8 mutants at position 146 and 17 mutants at position 145 displayed both a higher $(v_{Ps}/v_{Ph})_{ini}$, and a higher $[P_s]/[P_h]_{obs}$ and these values were nicely correlated (Fig. 2A, correlation coefficient = 0.75). An increase in $(v_{Ps}/v_{Ph})_{ini}$ was also correlated with the an elevated $[P_s]_{max}$, which appeared to be elevated for most of the selected mutants, ranging from 14% increase for mutant αF146G to 50% for mutant αF146Q (Fig. 2B). At position 146, a hydrophobic residue was favoured. Mutant αF146I, for instance, displayed a 4.6-fold elevation of the initial v_{Ps}/v_{Ph} as well as a higher $[P_s]_{max}$ (Table 2).

Mutant αR145W showed the highest $(v_{Ps}/v_{Ph})_{ini}$ value of 14.3, which is more than 10-fold better than the wild-type enzyme. Notably, the maximum amount of ampicillin that accumulated during the reaction was elevated for all αR145 mutants, ranging from 1.1-fold for mutant αR145A to 1.6-fold for mutants αR145S and αR145W. These results indicate that multiple mutations may lead to improvement of penicillin acylase and verify that the rapid screening method is suitable for identifying such improved mutants.

3.4. Kinetic properties of the best mutants and use of high substrate levels

The three mutants αR145L, αR145G and αR145S combined a strong increase in $(v_{Ps}/v_{Ph})_{ini}$ and $[P_s]_{max}$ with only a slight decrease of the synthetic activity (v_{Ps}) as compared to the wild-type enzyme. These mutants were selected for a more detailed analysis. The enzymes were purified to electrophoretic homogeneity and tested for their ability to synthesize ampicillin from 15 mM PGA and 25 mM 6-APA.

The maximum level of product accumulation ($[P_s]_{max}$) was 1.3–1.6-fold higher for the three mutants than for wild type (Table 4). The $(v_{Ps}/v_{Ph})_{ini}$ and P_{max} values were also strongly improved, which is in agreement with the results found with periplasmic extracts. The best values were found for mutants αR145S and αR145G, with a 4–5-fold higher $(v_{Ps}/v_{Ph})_{ini}$. The three αR145 mutants also displayed a remarkable improvement in β_0 and γ (Table 4), in agreement with the higher $(v_{Ps}/v_{Ph})_{ini}$ over the whole 6-APA concentration range (Fig. 3).

Table 5
Steady state kinetic parameters for hydrolysis of ampicillin and D-phenylglycine amide (PGA)

Mutant	Ampicillin			PGA			α
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	
E. coli wt	37	3.6	10	36	27	1.3	7.7
αR145L	20	1.9	11	6.8	9.1	0.75	15
αR145G	23	5.2	4.4	3.4	23	0.15	29
αR145S	14	4.6	3.0	2.7	13	0.21	14

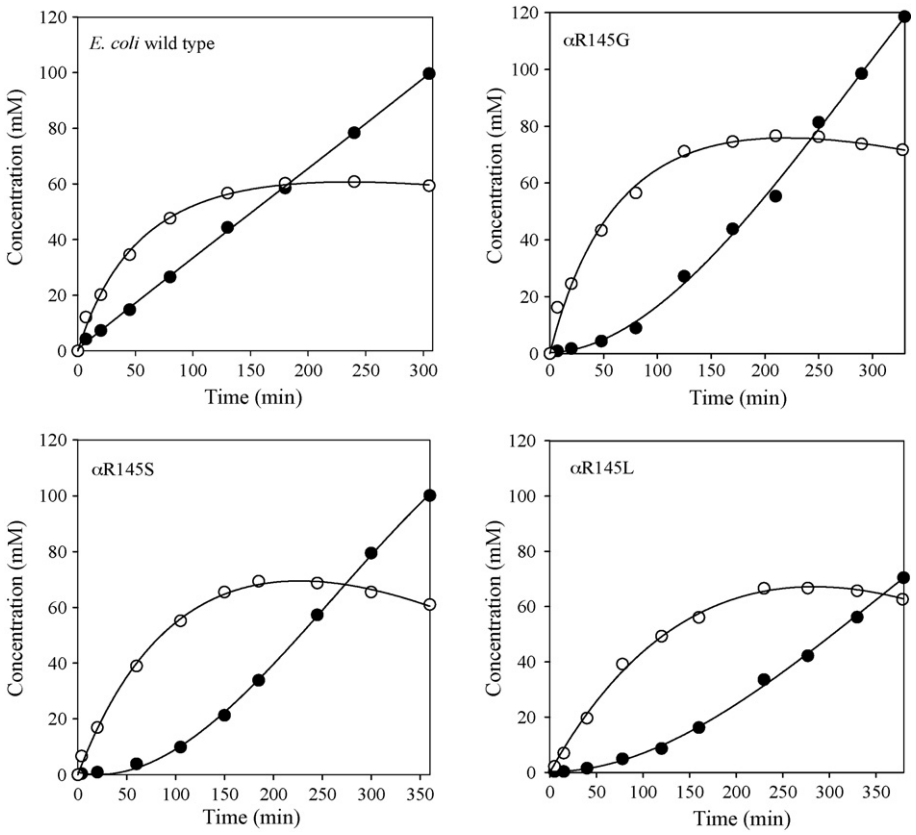

Fig. 4. Ampicillin synthesis catalysed by wild-type and mutant penicillin acylases. Reaction conditions: 0.3 M D-PGA, 0.1 M 6-APA, pH 6.3, 25 °C. Symbols: (●) ampicillin and (◯) D-phenylglycine.

The improvement (reduction) of γ is more than two-fold for mutant αR145L and more than three-fold for mutant αR145G.

The α values were determined by initial rate measurements with substrate and product (Table 5). A high α-value indicates preference for β-lactam product instead of acyl donor, which is unwanted. The k_{cat} and the specificity constant (k_{cat}/K_m) for PGA were significantly reduced for all mutants, indicating that formation of the acyl enzyme from the acyl donor is reduced compared to the wild-type enzyme. The specificity for ampicillin was also decreased for two of the three mutants, but this reduction was less drastic, resulting in an increased α value for all mutants. A high α indicates that the enzyme reacts with the acyl-transfer product preferentially over the acyl donor, which is not attractive for synthetic yield. A four-fold increase of α was observed for mutant αR145G, whereas there was a two-fold increase for the other two mutants.

As outlined above, the three kinetic parameters α, β and γ, govern the effectivity of PA-catalyzed ampicillin synthesis in a wide range of acyl donor (PGA) and nucleophile (6-APA) concentrations. Kinetic modelling showed that all three mutants can perform improved synthesis of ampicillin from PGA and 6-APA at high industrially relevant substrate concentrations (data not presented). We have tested this prediction experimentally and compared the ability of wild-type PA and the mutants to synthesize ampicillin at high substrate concentrations (Fig. 4). Mutant αR145G demonstrated a 16% increase in ampicillin yield over wild-type PA, reaching 77% conversion of 6-APA to ampicillin. The other two mutants (α145S and α145L) also showed a more effective conversion of 6-APA to ampicillin than the wild-type enzyme, which is in good agreement with the modelling.

4. Discussion

Penicillin acylase of *E. coli* is capable of transferring the acyl group of amides or esters to 6-APA or 7-ADCA, yielding semi-synthetic β-lactam antibiotics. Since the catalytic properties of this enzyme are non-ideal for the industrial preparation of such semi-synthetic β-lactams, there is a need for PAs with better synthetic properties. Here, we have used a structure-based approach to obtain such penicillin acylase mutants with improved catalytic properties. In 1995 the crystal structure of PA of *E. coli* was solved (Duggleby et al., 1995), and in recent years several papers reported that the enzyme was found in two well-defined conformations (Done et al., 1998; McVey et al., 2001; Alkema et al., 2000, 2002b). Upon binding of larger substrates, such as penicillin G, residues αR145 and αF146 move away from the acyl binding site according to an induced fit event. Residue αF146 seemed have van der Waals interactions with the thiazolidine ring of the substrate, and the crystal structures showed that residue α145R is bonded with one of the oxygens of the carboxylate of the leaving group bridged by two or three water molecules. For these reasons, residues αR145 and αF146 were selected as targets for random mutations.

We have used a fast screening method for PA-catalyzed ampicillin synthesis, involving automated liquid handling, incubation with enzyme, and HPLC injection. For detecting improved synthesis, the value $[P_s]/[P_h]_{obs}$, which represents the apparent efficiency of use of acyl donor for acylation versus hydrolysis, was used as criterion. Validation of the screening method showed that the correlation coefficient between the $[P_s]/[P_h]_{obs}$ and the $(v_{Ps}/v_{Ph})_{ini}$, which represents the ratio between the initial rate of synthesis and the initial rate of hydrolysis and is an intrinsic kinetic parameter of interest, of the αR145 mutants was 0.75. Indeed, 96% of the αR145 mutants that displayed a higher $[P_s]/[P_h]_{obs}$ also showed an increased $(v_{Ps}/v_{Ph})_{ini}$ value, confirming the practicality of rapid screening method.

Our results indicate that the best performing αR145 mutants, αR145G, αR145S and αR145L, have significantly improved synthetic properties over wild-type PA at high substrate concentrations, which leads to an increased conversion of 6-APA to ampicillin by up to 16% as well as to a decreased hydrolysis of the acyl donor by 29–56%. Thus, a single mutation in the targeted region can enhance the catalytic properties of the enzyme for antibiotic synthesis. The water-bridged hydrogen bonding observed in the X-ray structure between αR145 and the thiazolidine ring carboxylate apparently is not essential for PA-catalyzed ampicillin synthesis. Mutant αR145G showed the best parameters for the synthesis of ampicillin. It was able to produce 77 mM of ampicillin with a 29% reduction in the loss of the acyl donor due to hydrolysis as compared to the wild-type enzyme. To our knowledge, the three described αR145 mutants are the best published enzymes for ampicillin synthesis at industrially relevant conditions so far.

The improved synthetic performance of the best mutant enzymes was mainly due to a better (reduced) γ parameter and an increased β_0 parameter, which, respectively, indicate that in the mutants the acyl–enzyme saturated with β-lactam nucleophile is less sensitive to hydrolysis by water, and that the reactivity of the covalent acyl–enzyme with β-lactam nucleophile has improved. The latter effect, the increased β_0 value, as reflected in the dramatic increase of the $(v_{Ps}/v_{Ph})_{ini}$ ratios (Tables 3 and 4), can theoretically be due to improved binding of nucleophilic β-lactam nucleus to the acyl–enzyme or to a higher relative rate of deacylation by the nucleophile, as compared to water, after the nucleophile is bound in the active site of the acyl enzyme. The lumped steady state parameter β_0 does not differentiate between these possibilities. Nevertheless, in combination the positive kinetic effects on synthesis caused by improved γ and β_0 values were more important than the negative effects of the mutations on the preference of the mutated penicillin acylase for the β-lactam product over acyldonor. Preference for the produced antibiotic increased as indicated by the higher α value, mainly due to a decreased k_{cat} for phenylglycine amide (Table 5). The combined effects of the mutations caused higher levels of ampicillin accumulation and reduced loss of acyldonor by unproductive hydrolysis.

The ability of PA to catalyze ampicillin synthesis appeared very sensitive towards mutation of residues αR145 and αF146, and both positive and negative effects were observed. However, it is hard to predict what the precise effect of changing the selected residues will be, or provide a detailed structural explanation of the observed kinetic changes, and there is no simple correlation between certain properties of the introduced amino acid and the performance of the mutant enzyme. Therefore, a semi-random mutagenesis approach with adequate screening offers advantages over site-directed mutagenesis with introduction of only a few amino acids, which also hints at the limitations of error-prone PCR as compared to saturation mutagenesis at selected positions.

Together with the results of other studies (Alkema et al., 2002a; Gabor and Janssen, 2004; Wang et al., 2007) it appears that three positions (αR145, αF146 and βF24 in the *E. coli* sequence) are especially good targets for improving the catalytic properties of various penicillin acylases. The importance of these positions is also indicated by our earlier work with *E. coli* PA variants mutated at βF24 (Alkema et al., 2002a), and studies with a PAS2 penicillin acylase library mutated at the corresponding three positions (Gabor and Janssen, 2004). The latter mutant library was created from a PA gene obtained from an environmental gene library (Gabor and Janssen, 2004). A restricted set of mutations targetting these three positions in the penicillin acylase gene was recently explored by Wang et al. (2007), using the PA from *Bacillus megaterium*. The residues explored in their study were αY144, αF145 and βV24, which correspond to αR145, αF146 and βF24 of *E. coli* PA when a model of the *B. megaterium* PA is aligned with the *E. coli* PA structure. In the work of Wang et al. (2007) mutants with improved cephalexin synthetic activity were found, and especially a αY144R + βV24F double mutant performed well. All these data indicate that the selection of positions to be targeted in mutagenesis studies can be based on X-ray structures, molecular models if no structure is available, or results of experiments with a homologous enzyme, but the kinetic outcome is highly dependent on the specific enzyme that is used and the type of conversion that is studied. Since the best enzyme for synthetic applications is strongly dependent on the type of conversion (type of acyl donor and β-lactam nucleus) we foresee that different penicillin acylase variants optimized by directed evolution or protein engineering for specific applications can be developed.

The use of structural information to guide and focus mutagenesis during the creation of a library is also important in cases where high-throughput screening is difficult because of the nature of the activity that is under study. Since the synthetic performance is dependent on the substrates, we judge it preferable to use structure-inspired mutagenesis (Gabor and Janssen, 2004) to increase the frequency of mutants with desirable properties in a library, and then use medium-throughput screening with the real conversion, as compared to high-throughput screening with (chromogenic or fluorescent) model substrates instead of the real substrates.

Ampicillin synthesis using the obtained mutants can likely be further improved by a rational biocatalytic process design using different optimization approaches described in the literature: medium engineering (Rosell et al., 1998; Park et al., 2000; Youshko et al., 2002b; Ferreira et al., 2004), increasing the initial concentrations of acyl donor (PGA) and penicillin nucleus (6-APA) (Youshko et al., 2000), repetitive addition of substrates

to the reaction mixture (Youshko et al., 2001), adequate immobilization of the enzyme (Mateo et al., 2002; Kallenberg et al., 2005) or removal of product in two-phase systems (Hernandez-Justiz et al., 1998; Terreni et al., 2005).

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